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(54) Title: METHOD OF POLYPEPTIDE RENATURATION

(57) Abstract: The present invention relates to a method for producing a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises: (i) expressing the polypeptide in a bacterium or as a non-secreted polypeptide in yeast; (ii) extracting the expressed polypeptide from the bacterium or yeast and denaturing the polypeptide; (iii) refolding the extracted polypeptide in the presence of arginine and urea; and optionally (iv) recovering the refolded polypeptide.

# METHOD OF POLYPEPTIDE RENATURATION

## Field of the Invention

The present invention relates to a method of producing malaria polypeptides in a bacterium or yeast.

## Background of the invention

Malaria is a major public health problem in many parts of the tropical world with millions of deaths occurring annually from this disease. Various species of *Plasmodium* are responsible for malaria including *Plasmodium falciparum* and *Plasmodium vivax* and as drug resistant strains of these pathogens have emerged there is an urgent need for the development of vaccines that can provide effective protection against them.

One potential target for vaccines against malaria is to prevent the entry of the parasite into the erythrocyte. The invasion of erythrocytes by the parasite is mediated by specific interactions between receptors on the erythrocyte and ligands on the parasite (C. E. Chitnis *et al*, 1999. In Malaria: Molecular and Clinical Aspects. Ed. P. Perlmann and M. Wahlgren. Harwood Academic Publishers. p249-285). The ligands on the parasite responsible for this interaction belong to a family of erythrocyte binding proteins known as the EBP family and these include the Duffy binding protein of *Plasmodium vivax* (PvDBP) and the 175kd erythrocyte binding antigen of *Plasmodium falciparum* (EBA-175).

The members of the EBP family share similar structural features. The extracellular domains of EBP family members contain two conserved cysteine rich regions, regions II and VI, at their amino and carboxyl ends respectively. The functional receptor-binding domain of PvDBP has been mapped to region II (Chitnis C. E. and Miller L. H. , 1994, J. Ex. Med. **180**, p497-506.). In EBA-175, which contains a tandem duplication (F1 and F2) of the N-terminal cysteine- rich region, the functional binding domain maps to the F2 region (Sim B. K. L. *et al*, 1994 *Science*. **264**, p1941-1944). The binding domains of the PvDBP and EBA-175

proteins are therefore known as PvRII and PfF2 respectively. PvRII binds the Duffy antigen on human erythrocytes whilst PfF2 binds sialic acid residues of glycophorin A on human erythrocytes.

The binding domains PvRII and PfF2 belong to a family of conserved cysteine-rich domains that are referred to as Duffy-binding-like (DBL) domains because the first binding domain to be identified was derived from the *Plasmodium vivax* Duffy binding protein. Other members of the DBL family include the cysteine-rich domains found in the extracellular region of the PfEMP-1 family of proteins from *Plasmodium falciparum* (Baruch *et al*, 1995, *Cell.* **82**, p77-88; Smith *et al*, 1995, *Cell.* **82**, 101-110; and Su *et al*, 1995. *Cell.* **82**, p89-100). Some members of the PfEMP-1 family bind specific endothelial receptors such as ICAM-1, chondroitan sulfate A (CSA), hyaluronic acid (HA) and CD31 to mediate cytoadherence of *Plasmodium falciparum*-infected trophozoites and schizonts to the vascular endothelium (C. E. Chitnis *et al*, 1999. In Malaria: Molecular and Clinical Aspects. Ed. P. Perlmann and M. Wahlgren. Harwood Academic Publishers. p249-285). Binding to ICAM-1 in brain capillaries is implicated in the pathology of cerebral malaria and binding to CSA and HA is implicated in sequestration in the placenta, which may lead to complications in pregnancy (Turner *et al*, 1994, *Am. J. Pathol.* **145**, p1057-1071; Fried *et al*, 1996. *Science* 272, p1502-1504; and Beeson *et al*, 2000. *Nature Medicine* **6**, p86-90). Functional DBL domains from PfEMP-1 that mediate binding to ICAM-1 and CSA have been identified and are being developed as vaccines with the goal of eliciting cytoadherence-blocking antibodies (Smith *et al*, 2000, *PNAS*, **97**, p1766-1771; and Buffet *et al*, 1999 *PNAS*, **96**, p12743-12748).

The binding domains of EBP family members and in particular PvRII and PfF2 are promising candidates for vaccines against *Plasmodium vivax* and *Plasmodium falciparum*. Antibodies capable of binding PfF2 block invasion of erythrocytes by *Plasmodium falciparum* and may provide protection against *Plasmodium falciparum* (Narum *et al*, 2000, *Infection and Immunity*, **68**, p1964-1966). However, in order for the binding domains to elicit protective antibodies which will block erythrocyte binding and invasion it is necessary for them to be in

their native conformations.

Although the PfF2 domain has been expressed in baculovirus and the expressed protein was thought to adopt its native conformation the proteins expressed were glycosylated. Malaria parasite proteins are not normally glycosylated. Glycosylation may mask essential epitopes of the protein. This masking may prevent vaccines comprising the recombinant proteins from raising a protective immune response against the malarial parasite. Furthermore, although DBL domain containing proteins can be expressed in baculovirus and mammalian systems it is expensive to do so and yields of protein are relatively poor.

The expression of PvRII and PfF2 in bacteria, or as a non secreted polypeptide in yeast, produces recombinant proteins which are not glycosylated and production in bacteria or yeast is less expensive than expression in baculovirus or mammalian systems. However, expression of proteins containing disulfide bridges, which DBL domains contain, in bacteria or as non-secreted proteins in yeast, often results in the accumulation of incorrectly folded biologically inactive protein in insoluble inclusion bodies and expression of the PfF2 and PvRII domains leads to the information of inclusion bodies. This incorrectly folded protein is not suitable for use in a vaccine and although methods to renature proteins recovered from inclusion bodies are known none have been developed for refolding of DBL domains.

Summary of the Invention

The present invention provides a method for producing a polypeptide comprising a Duffy binding like (DBL) domain in bacteria or yeast. When proteins comprising DBL domains are expressed in bacteria they accumulate in incorrectly folded, biologically inactive forms in insoluble inclusion bodies. When proteins containing DBL domains are expressed in yeast as non-secreted polypeptides they also form inclusion bodies. The incorrectly folded polypeptide is not suitable for use in vaccines or for other purposes where it is necessary that the polypeptide adopts a biologically active conformation. The present inventors have shown, for the first time, how the accumulated DBL domain containing recombinant polypeptide can be

recovered from the inclusion bodies and refolded into a biologically active form.

The inventors have shown that, after recovery of the recombinant polypeptide from the inclusion bodies and denaturation, the polypeptides can be refolded by rapid dilution in the presence of urea and arginine so that they adopt a biologically active conformation. The inventors have further demonstrated that after refolding, if the arginine is removed prior to the removal of the urea that the yield of refolded polypeptide achieved is maximised.

One advantage of the method of the present invention is the polypeptides obtained are non-glycosylated. DBL domain containing polypeptides are not naturally glycosylated. Although methods for obtaining biologically active DBL domain containing polypeptides are known in the art these use baculovirus or mammalian cell systems to express the polypeptide and the recombinant polypeptides obtained are glycosylated. Glycosylation may mask important epitopes of the polypeptide and prevent vaccines containing these polypeptides from raising a protective immune response. A further advantage of the present method over those systems using baculovirus or mammalian cells for expression is that it is far less expensive.

Accordingly the present invention provides a method of producing a cysteine rich polypeptide. In particular, the present invention provides a method of producing a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises:

(i) expressing the polypeptide in a bacterium, or as a non-secreted polypeptide in a yeast;

(ii) extracting the polypeptide from the bacterium or yeast and denaturing the polypeptide;

(iii) refolding the extracted polypeptide in the presence of arginine and urea; and optionally

(iv) recovering the refolded polypeptide.

The present invention further provides

- a pharmaceutical composition obtainable or obtained by a method of

the invention; and

- a vaccine composition obtainable or obtained by a method of the invention.

The invention also provides a method for the identification of a substance that modulates the interaction between a polypeptide produced by a method of the invention and a host cell receptor involved in the entry of a parasite into a host cell, wherein the method comprises:

(i) contacting the receptor with the polypeptide in the presence of a test substance;

(ii) determining the effect of the test substance on the interaction between the receptor and polypeptide thereby to determine whether the test substance is capable of modulating the interaction between the receptor and polypeptide.

The invention further provides a method of treating or preventing malaria in an individual comprising immunising the individual with a pharmaceutical composition or a vaccine of the invention.

Brief Description of the Figures

**Figure 1. Purification and refolding of recombinant PvRII expressed in *E. coli*. A. Coomasie stained SDS-PAGE gel.** Molecular weight standards (MW); whole cell extract (WC); inclusion bodies (IB); elute fractions after purification by metal affinity chromatography (Ni), ion-exchange chromatography (IEX) and gel permeation chromatography (GPC). **B. Western blot.** Rabbit antiserum raised against a 43 amino acid peptide derived from PvRII was used to detect PvRII. Molecular weight standards (MW); whole cell extract (WC); inclusion bodies (IB); elute fractions after purification by metal affinity chromatography (Ni), ion-exchange chromatography (IEX) and gel permeation chromatography (GPC).

**Figure 2. Purification and refolding of recombinant PfF2 expressed in *E. coli*. A. Coomasie stained SDS-PAGE gel.** Molecular weight standards (MW);

whole cell extract (WC); inclusion bodies (IB); elute fractions after purification by metal affinity chromatography (Ni), ion-exchange chromatography (IEX) and gel permeation chromatography (GPC). **B. Western blot.** A mouse monoclonal antibody raised against 5-His was used to detect PfF2, which contains a 6-His fusion at the C-terminus.

**Figure 3. Silver-stained SDS-PAGE gels of refolded, purified PvRII (A) and PfF2 (B).** Different amounts (0.6 μg, 1 μg, 2 μg) of refolded and purified recombinant PvRII and PfF2 were separated by SDS-PAGE and detected by silver staining to determine levels of purity.

**Figure 4. Mobility of refolded PvRII (A) and PfF2 (B) before and after reduction.** Refolded PvRII and PfF2 have slower mobility by SDS-PAGE after reduction with dithiothreitol (+DTT) indicating the presence of disulfide linkages.

**Figure 5. RP-HPLC profile of refolded, purified PvRII (A) and PfF2 (B).** Refolded, purified PvRII and PfF2 were analyzed by reverse phase chromatography on a C8 column. Both elute as single, symmetric peaks indicating that they contain a single, homogeneous population of conformers.

**Figure 6. Gel filtration chromatography profile of refolded and purified PvRII (A) and PfF2 (B).** Both PvRII and PfF2 elute as single peaks with the expected mobility on a Superdex 75 gel filtration column (Pharmacia). Elution times of protein standards of known size (1, bovine serum albumin 66 kD; 2, carbonic anhydrase 29 kD; 3, cytochrome C 12.4 kD; 4, aprotinin 6.5 kD) are shown.

**Figure 7. CD spectra of refolded PvRII and PfF2. A. CD spectra of denatured and refolded PvRII. B. CD spectra of refolded PvRII and PfF2.**

**Figure 8. Erythrocyte binding assay with refolded and purified PvRII (A) and PfF2 (B). A. Binding specificity of refolded PvRII.** Refolded PvRII (RF) binds human Duffy positive (Fya+b+) but not human Duffy negative (Fya-b-) erythrocytes. **B. Binding specificity of refolded PfF2.** Refolded PfF2 (RF) binds human (Hu) and rhesus (Rh) erythrocytes but not neuramindase-treated (Neu) human and rhesus erythrocytes.

**Figure 9. Reactivity of rabbit antisera raised against refolded, purified**

**PvRII and PfF2 as determined by ELISA. A. Rabbit antisera raised against PvRII.** Reactivity of rabbit antisera raised against refolded PvRII as determined by ELISA. **B. Rabbit antisera raised against PfF2.** Reactivity of rabbit antisera raised against refolded PfF2 as determined by ELISA.

**Figure 10. Detection of inactive PvRII by immunoprecipitation and Western blot following pre-absorption with Duffy positive erythrocytes.** Refolded PvRII was immunoprecipitated, separated by SDS-PAGE and detected by Western blot either without pre-absorption (-) or after pre-absorption with Duffy positive (D+) or Duffy negative (D-) human erthrocytes.

**Brief Description of the Sequences**

SEQ ID NO: 1 shows the amino acid sequence of the PvRII domain of the PvDBP protein of *Plasmodium vivax.*

SEQ ID NO: 2 shows the amino acid sequence of the PfF1 domain of the EBA-175 of *Plasmodium falciparum.*

SEQ ID NO: 3 shows the amino acid sequence of the PfF2 domain of the EBA-175 of *Plasmodium falciparum.*

SEQ ID NOs: 4 and 5 show the nucleotide sequences of the primers used to amplify the PvRVII domain of PvDBP in the Example.

SEQ ID NOs: 5 and 6 show the nucleotide sequences of the primers used to amplify the PfF2 domain of EBA-175 in the Example. SEQ ID NO: 7 is a further primer sequence.

Detailed description of the Invention

***DBL domain containing polypeptides***

The present invention provides a method for producing a polypeptide comprising a DBL domain. Typically DBL domains are conserved, cysteine-rich domains that share significant homology with region II, the erythrocyte binding domain of the *Plasmodium vivax* Duffy binding protein. Region II of PvDBP (PvRII) contains 12 cysteines. DBL domains from other erythrocyte binding proteins

such as EBA-175 usually have these 12 conserved cysteines and in addition may have others not present in PvRII. DBL domains from PfEMP-1 usually contain the first 10 cysteines of PvRII and in addition may contain others. Besides the conserved cysteines other short conserved amino acid stretches containing hydrophobic amino acid residues may be shared by DBL domains. Sequence homology between different DBL domains is typically in the range of 20-90%.

Preferably the polypeptide will be from a pathogen and in particular from a parasite. In a preferred embodiment of the invention the parasite will be one which is responsible for malaria. The polypeptide may be derived from a drug resistant strain of a particular pathogenic or parasitic species. In a particularly preferred embodiment of the invention the polypeptide will be one from *Plasmodium falciparum* or *Plasmodium vivax*.

In a preferred embodiment of the invention the polypeptide will be a parasite protein involved in the binding of the parasite to host cells and in particular to host erythrocytes or vascular endothelium. Examples of polypeptides which may be used include the binding domains (DBL domains) of the PvDBP protein of *Plasmodium vivax*, the EBA-175 protein and the PfEMP-1 family of proteins of *Plasmodium falciparum*. The polypeptide may be a homologue of any of these proteins and in particular a homologue of EBA-175 encoded by *Plasmodium falciparum*. Examples of preferred PfEMP-1 family proteins are those responsible for binding to vascular and in particular brain, placental or foetal vascular endothelium and these include those responsible for binding to ICAM-1, chondroitan sulfate A (CSA), hyaluronic acid (HA) and CD31. Also included are homologs of these proteins from other pathogenic species and in particular from other species of parasites which include a DBL domain and are responsible for binding of the parasite or pathogen to host cells.

The DBL domain containing polypeptide may be a fragment or a variant of a parasitic polypeptide and in particular of those parasite polypeptides responsible for binding to a host cell. Preferably the fragment or variant will be capable of specifically binding to the host cell or host cell receptor for the parasite. Particularly preferred fragments include those of the above mention proteins and in particular the

PvRII domain of PvDBP, the PfF2 domain of EBA-175 and a domain from PfEMP-1 family protein responsible for receptor binding. Also included are portions of these domains containing a DBL domain and preferably capable of binding a host cell receptor.

In a preferred embodiment of the invention the polypeptide will have the amino acid sequence of SEQ ID NOs: 1 to 3 or be a fragment or variant of these sequences. Preferably the fragment or variant will be able to bind the Duffy antigen (for the variants and fragments of SEQ ID NO: 1) or Glycophorin (for the variants or fragments of SEQ ID NOs: 2 and 3). Typically the polypeptide will comprise from 4 to 14 cysteine residues, preferably 8 to 12 cysteine residues and more preferably 10, 11 or 12 cysteine residues.

The term "variant" refers to a polypeptide which has the same essential character or basic biological functionality as a DBL domain. Typically the variant can bind a host cell receptor, preferably a receptor for a parasite polypeptide, more preferably a host cell receptor for a malaria polypeptide and in particular a host cell receptor for a *plasmodium falciparum* or *plasmodium vivax* polypeptide. In an especially preferred embodiment of the invention the variant can bind the Duffy antigen or glycophorin.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example according to Table I. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

**Table I**

| | | |
|---|---|---|
| ALIPHATIC | Non-polar | G A P |
| | | I L V |
| | Polar-uncharged | C S T M |
| | | N Q |
| | Polar-charged | D E |
| | | K R |
| AROMATIC | | H F W Y |

Shorter polypeptide sequences are within the scope of the invention and particular fragments of the above mentioned polypeptides. For example, a peptide of at least 20 amino acids or up to 50, 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as it is able to bind a host cell receptor which binds a parasite polypeptide. In particular, but not exclusively, this aspect of the invention encompasses the situation when the protein is a fragment of the complete protein sequence of PvDBP, EBA-175, homologues of the EBA-175 protein and PfEMP-1 family polypeptides

Typically, polypeptides with more than about 20% identity preferably at least 30% or at least 50% and particularly preferably at least 70% at least 90% or at least 99% identity, with the amino acid sequences of SEQ ID NOs: 1 or 3, or of the DBL domains contained therein are considered as variants of the polypeptides within the meaning of the invention. Such variants may include allelic variants and the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the peptide maintains a basic biological functionality of the DBL domain.

The invention also includes homologues of the above mentioned polypeptides and in particular those from parasitic or pathogenic species, preferably those from malaria parasites and in particular those from *plasmodium vixax* and *plasmodium falciparum*. Homologues will preferably be able to bind a host cell receptor for a parasite/pathogen, preferably a receptor involved in binding a malaria parasite and in particular a receptor involved in binding a *plasmodium vixax* or *plasmodium falciparum* polypeptide.

Software for performing BLAST analyses and identifying homologues is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, 1990). These initial

neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Any combination of the above mentioned degrees of sequence identity and minimum sizes may be used to define polypeptides of the invention, with the more stringent combinations (i.e. higher sequence identity over longer lengths) being preferred.

The DBL domain containing polypeptides may contain heterologous regions. For example the polypeptides may contain members of a specific binding pair to allow their purification. Typically this will be a purification tag which as used herein is an amino acid sequence that can bind to a target under suitable conditions. Usually the target will be provided by a manufacturer, who will recommend a purification tag and suitable conditions. The member of the specific binding pair may

be present anywhere in the polypeptide but is preferably present at the N or C terminus of the polypeptide and may be removed by cleavage.

In a preferred embodiment, the purification tag is a His-Tag. Typically the His-tag comprises multiple contiguous histidine residues, preferably from 3 to 20, more preferably from 4 to 10 most preferably 6. Typically the His-tag will be positioned at either or both of the N- and C- terminals of the fusion polypeptide. The preferred target for the His-tag is NiNTA, which is available commercially, e.g. from Qiagen (Germany). Other preferred purification tags include a self-cleaving chitin-based ligand system, a sequence capable of binding maltose, a nickel-based ligand system and a glutathione S-transferase based system.

If the polypeptide is for use in an assay it may be a fusion polypeptide with an enzyme, transcription factor domain, green fluorescent protein or other suitable reporter sequence.

The polypeptide may also comprises a cleavage site allowing release of other regions of the polypeptide from the DBL domain for example the purification tag. A cleavage site is any amino acid sequence that facilitates cleavage of fusion polypeptide to release the polypeptide of interest.

***Polypeptide expression***

Typically, the nucleotide sequences encoding the DBL domain containing polypeptide, fragment or variant will be inserted into a vector, introduced into a suitable host and expressed therein. Typically a prokaryotic bacterium, and in particular a gram negative bacterium, will be used as the host cell. In an especially preferred embodiment of the invention the bacterium used will be *E.coli*. Alternative prokaryotic bacteria and expressions may be used such as, for example *Lactobacillus* or *Bacillus subtilis*. In one embodiment of the invention the polypeptide may be expressed as a non-secreted protein in yeast strains such as *Saccharomyces cerivisae* or *Pichia pastoris*. This is because in yeast, proteins are not glycosylated if they are not targeted for secretion.

Typically the polypeptide will include a purification tag to allow its recovery.

Suitable cloning methods, vectors, bacteria or yeast strains and methods for expression of polypeptides in bacteria or yeast are well understood by persons skilled in the art, and include techniques such as those disclosed in Sambrook *et al*, Molecular Cloning: a Laboratory Manual, 2nd Edition, CSH Laboratory Press, 1989, the disclosure of which is included herein in its entirety by way of reference. After expression of the recombinant polypeptide typically the polypeptide will accumulate in inclusion bodies and these are then recovered from the bacterium or yeast cell. Typically the recombinant polypeptide is recovered using a method as follows:

- the bacteria or yeast are harvested, the cells lysed, typically by sonication, and the inclusion bodies recovered by centrifugation;
- the inclusion bodies are solubilised, preferably in guanidine-HCl at a concentration of from 4 to 8 Molar and in particular at a concentration of 6 Molar;
- insoluble debris are removed; and
- the recombinant polypeptide is recovered and retained in denaturing conditions before refolding.

In a preferred embodiment the buffer for the solubilisation of inclusion bodies contains 10mM Tris HCl, pH 8.0, 100mM $NaH_2Po_4$, 100mM NaCl , 6M guanidine–HCl and 10mM DTT. Typically the solubilisation is carried out from 5 to 24 hours, preferably 8 to 12 hours and typically with constant stirring. Preferably the solubilisation is carried out at a temperature from 15 to 25°C, more preferably from 20 to 25°C and in particular at 23 °C.

If the polypeptide is His tagged it may be recovered using metal affinity chromatography and typically a Ni-NTA column.

***Refolding of the polypeptide***

After the polypeptide is recovered from the inclusion bodies it is typically refolded by dilution in the presence of arginine and urea. Typically before dilution the polypeptide is at a concentration of at least 1 mg/ml and preferably at a

concentration of at least 4mg/ ml. The solution containing the polypeptide is then diluted using a solution containing arginine and urea. Preferably the dilution is a rapid dilution typically performed by adding the solution containing the polypeptide to the other solution rather than *vice versa*.

Typically the polypeptide is diluted with the solution by a factor of 50 to150 fold, preferably from 75 to125 fold and more preferably between 90 and 100 fold. In a particularly preferred embodiment of the invention the dilution factor used is 100 fold. Typically, the polypeptide is diluted to a concentration of from 10 to 100µg/ml, preferably to a concentration of from 30 to 70 µg/ml and more preferably from 40 to 60 µg/ml. In a preferred embodiment of the invention the polypeptide is diluted to a concentration of 45 µg/ml.

Typically the ratio of arginine to urea in the solution used for dilution is from 1:1 to 1:5, preferably from 1:1 to 1:3 and more preferably the ratio is 1:2. Preferably the urea is present at a concentration of from 0.5 to 1.5 M, more preferably from 0.7 to 1.2 M and even more preferably from 0.9 to 1.1 M. Preferably, the arginine is present at a concentration of from 0.1 to 1 M, preferably from 0.3 to 0.7 M and more preferably between 0.4 and 0.6 M. In a particularly preferred embodiment of the invention the urea is present at a concentration of 1.0 M and/or the arginine is present at a concentration of 0.5 M.

The pH of the solution may be from pH 4.0 to pH 9.0, preferably from pH 4.0 to pH 7.0 and more preferably from pH 4.0 to pH7.0. The pH used will depend on the particular protein and the optimal pH and other conditions may be readily determined by titrating the conditions against the yield of correctly folded protein obtained.
In a preferred embodiment of the invention the rapid dilution solution in addition to arginine and urea also comprises 50mM phosphate buffer - (pH 7.2 for PvRII and pH 5.8 for PfF2), reduced glutathione (GSH) at a concentration from 0.2 mM to 2 mM and oxidised glutathione (GSSG) at a concentration of from 0.02 mM to 0.2 mM. The ratio of reduced glutathione to oxidised glutathione is preferably from 5:1 to 25: 1, more preferably from 8:1 to 15:1 and even more preferably from 10:1 to 15:1. In a preferred embodiment of the invention the ratio will be 10:1.

In an especially preferred embodiment of the invention the refolding is carried out in a buffer comprising 1mM reduced glutathione (GSH), 0.1mM oxidised glutathione (GSSG), 1M urea and 0.5M arginine.

Typically the refolding is carried out at a temperature from 5 to 37°C, preferably at from 5 to 20°C and more preferably from 5 to 15°C. In a particularly preferred embodiment of the invention the refolding is carried out at 10°C. Typically the refolding step is carried out for a period of from 2 to 64 hours, preferably from 12 to 48 hours, more preferably from 24 to 36 hours. In a preferred embodiment of the invention the refolding step is carried out at a temperature of from 5 to 15°C and for a period of from 12 to 48 hours. In a particularly preferred embodiment the refolding step is carried out at a temperature of 10°C and for a period of 36 hours.

Multiple additions of the denatured, extracted polypeptide may be made to the refolding solution. This allows refolding larger amounts of polypeptide without loss of yield of refolded polypeptide. Preferably such multiple additions will be made with intervals of ½ to 3 hours, more preferably 1 to 2 or 1½ to 2½ hours, e.g. approximately 2 hours. It is preferred to make 2, 3, 4 or 5 additions, more preferably 3 additions. Desirably, a final concentration of peptide of 40 to 80µg/ml, e.g. 50 to 70µg/ml, preferably approximately 60µg/ml will be achieved in the refolding solution, either by a single addition of peptide, or preferably by multiple additions as just described. In a preferred embodiment of the invention denatured polypeptide is added three times with intervals of approximately 2 hours between additions to achieve a final concentration of approximately 60µg/ml.

***Removal of arginine and urea from the refolded polypeptide***

After refolding the polypeptide is recovered. The recovery process may involve the removal of the arginine and urea. As the present inventors have demonstrated that removing the arginine prior to the removal of the urea substantially increases the yield of correctly refolded protein the recovery process will more preferably include removal of arginine and then subsequently the removal of the urea.

The removal of arginine is typically carried out by dialysis. Typically the dialysis is carried out for from 12 to 72 hours, preferably from 24 to 48 hours and in particular it is carried out for 48 hours. The refolding solution is typically dialysed against a dialysis buffer comprising a phosphate buffer and urea. Preferably the concentration of urea in the dialysis buffer is equimolar or approximately equimolar with the concentration of urea in the solution containing the protein. Typically the dialysis buffer will comprise 50mM phosphate buffer at a pH appropriate to the particular protein (pH 6.5 for PvRII and pH 5.8 for PfF2) and urea at an equimolar concentration to the concentration of urea in the solution being dialysed.

The urea may be removed by a purification method such as ion exchange chromatography either separately or in combination with gel filtration chromatography. Typically if ion exchange chromatography is used the refolded protein is loaded onto an equilibriated sepharose column and after washing the bound protein eluted with a linear gradient of equilibration buffer containing NaCl Preferably fractions containing the protein of interest are then pooled and purified using gel filtration chromatography.

***Assessment of the biological activity of the polypeptides of the invention***

Preferably the refolded polypeptides of the invention are in their native conformation or in a conformation similar to their native conformation. Preferably the polypeptide is able to specifically bind its normal ligand on the host cell. It is especially preferable that polypeptides of the invention to be used in the vaccines or assays of the invention adopt their native conformation or a conformation closely approximating their native conformation. Preferably at least the regions of the polypeptide which interacts with and/or bind the host cell receptor will adopt their native conformation.

Assays such as reverse phase chromatography, circular dichroism analysis and crystallography may be used to assess whether the polypeptide has refolded and/or whether the refolded polypeptide obtained is present in a single or variety of conformations. Refolded polypeptides where the majority, and preferably all, of the

polypeptide is in a single, and preferably the native, conformation are especially preferred for use in the assays, vaccines and methods of the invention.

In a particular preferred embodiment of the invention the binding of the refolded polypeptide to a host cell or the isolated host cell receptor it is specific for will be assessed. Polypeptides which do bind to these are preferred for use in the vaccines or assays of the invention. Typically, in such assays, the binding of the polypeptide to cells, preferably erythrocytes, with the receptor and lacking the receptor will be compared.

Where the polypeptide is EBA-175 or a fragment, variant or homologue thereof the ability of the polypeptide to bind to glycophorin may be assessed. The ability of the polypeptide to bind to neuraminidase treated erythrocytes (which lack sialic acid residues) and untreated human erythrocytes may be used to determine if the polypeptide can specifically bind glycophorin in its normal state and hence normal human erythrocytes.

Where the polypeptide is PvDBP or a fragment, variant or homologue thereof the ability of the polypeptide to bind to the Duffy antigen may be determined by comparing the binding of the polypeptide to Duffy positive human erythrocytes and Duffy negative erythrocytes.

The suitability of particular refolded polypeptides for use in the assays and methods of the invention may also be determined by their ability to raise antibodies which block the binding of the native protein they are derived from to its receptor and/or prevent the entry of the parasite into the host cell. This may be done by raising anti-sera against the polypeptide in animals such as rabbits and then assessing whether the anti-sera can inhibit or prevent binding to the host cell receptor or the host cell itself. Polypeptides of the invention which can raise such anti-sera are especially preferred for use in the vaccines, assays and methods of the invention.

***Assays using the polypeptides of the invention***

The invention provides methods of identifying substances which can bind to a

polypeptide of the invention and more preferably can bind to the polypeptide and hence prevent the binding of the polypeptide to the host cell receptor for it.

In one embodiment of the invention the binding of the polypeptide to the host cell receptor is compared in the presence and absence of the test substance.

The host cell receptor may be in an isolated form or present on a cell. Preferably the receptor is a receptor associated with the entry of a malaria parasite, typically *Plasmodium falciparum* or *Plasmodium vivax*, into the host cell. In a preferred embodiment of the invention the receptor is the Duffy antigen or glycophorin. The cell may either naturally express the receptor or express the receptor as it has been transformed by a nucleic acid encoding the receptor. Preferably the cell is an erythrocyte. The binding of the polypeptide to cells with and without the receptor is typically compared in the presence and absence of the test substance. In cell based assays the actual entry of hte polypeptide inot the cell may be assessed.

In such methods the test substance may inhibit the binding of the receptor to the polypeptide either by the candidate substance binding the polypeptide or by the test substance binding the receptor. Thus the method can identify not only substances that bind the polypeptide, but also substances that act as mimics of the polypeptide.

Alternatively the binding of the test substance to the polypeptide is determined. Substances that are identified as able to bind the polypeptide may then be assessed to see if they can inhibit the binding of the polypeptide to its receptor. In the assays the polypeptide may be immobilised on a solid support or may be in solution. The binding of the test substance to the polypeptide or the binding of the polypeptide to the receptor may be determined by measuring a characteristic of the polypeptide or receptor that changes upon binding, such as spectroscopic changes. Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical entities and compounds, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display (e.g. phage display libraries) and antibody products.

Typically, organic molecules will be screened, preferably small organic molecules which have a molecular weight of from 50 to 2500 daltons.

Test substances may be used in an initial screen of, for example, 10 substances per reaction, and the substances of these batches which show inhibition of binding of the polypeptide to the receptor or which bind to the polypeptide tested individually. Test substances may be used at a concentration of from 1nM to 1000μmM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM. Preferably, the activity of a test substance is compared to the activity shown by a known inhibitor of receptor - polypeptide binding. A test substance which acts as an inhibitor may produce a 50% inhibition of activity of the receptor. Alternatively, a test substance which acts as an activator may produce 50% of the maximal activity produced using a known activator.

***Use of the polypeptides of the invention to make antibodies***

Antibodies to the refolded polypeptides produced using the method of the invention can be made by use of the following methods. An antibody to the substance may be produced by raising antibody in a host animal against the whole substance or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known.

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a

conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

***Composition and, vaccines using the polypeptides of the invention***

The polypeptides of the invention may be used in vaccines. Typically the vaccines will be used to prevent malaria. A polypeptide of the invention can therefore be used to prevent infection by *Plasmodium faciparum* or *Plasmodium vivax*. The vaccine may be in the form of a pharmaceutical composition which comprises the polypeptide and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. A pharmaceutically acceptable adjuvant such as alum may be present. Typically the administration is (and thus the composition is formulated for) parenteral, intravenous, intramuscular, subcutaneous, transdermal, intradermal, oral, intranasal, intravaginal, or intrarectal administration. Typically the vaccine is given 1, 2, 3 or more separate administrations, each of which is separated by at least 12 hours, 1 day, 2, days, 7 days, 14 days, 1 month or more.

An effective amount of the polypeptide is administered to a host, typically a human being. The dose of polypeptide administered as a vaccine may be determined according to various parameters, especially according to the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration

and dosage for any particular patient. A suitable dose may however be from 10 μg to 1g, for example from 100 μg to 50mg of the polypeptide. These values may represent the total amount administered in the complete treatment regimen or may represent each separate administration in the regimen.

Substances identified in the assays of the invention may be used to treat malaria. Such a substance may be used to treat infection by *Plasmodium falciparum* or *Plasmodium vivax*. In particular those substances identified which can bind to the polypeptides of the invention, preferably those that can prevent the binding of the polypeptide to its receptor and more preferably those which are capable of preventing invasion of the host cell by the parasite, will be used in the treatment of malaria. The condition of a patient suffering from malaria can thus be improved. The symptoms of malaria may be ameliorated.

Substances identified according to the screening methods outlined above may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art. For example, a suitable substance may be dissolved in physiological saline or water for injections. The exact nature of a formulation will depend upon several factors including the particular substance to be administered and the desired route of administration. Suitable types of formulation are fully described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Eastern Pennsylvania, 17th Ed. 1985, the disclosure of which is included herein of its entirety by way of reference.

The substances may be administered to a host, typically a human being, by enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intravenous, intra-arterial, intramuscular, intraperitoneal, topical or other appropriate administration routes. An effective amount of a substance is given to a patient. The amount that is administered will depend upon a number of factors such as the substance used; the age, weight and condition of the patient; the route of administration and the required regimen. A physician will be able to determine the required route of administration and the dosage for any particular patient. A typical amount adminstered may be from 10μg to 1g, for example from 100μg to 100mg.

The following Example illustrates the invention

**Example**

**Plasmid constructs for expression of PvRII and PfF2 in *E. coli*:** DNA encoding PvRII with a C-terminal six His fusion was amplified by PCR using primers 5'- GCA TGC CAT GGA TCA TAA GAA AAC GAT CT 3' and 5' - CGA GTG TCG ACT CAG TGA TGG TGA TGG TGA TGT GTC ACA ACT TCC TGA GT - 3' and a plasmid containing the gene encoding *Plasmodium vivax* Duffy binding protein as template. The PCR product was restricted with NcoI and SalI and cloned in the vector pET28a+ (Novagen Inc., USA) downstream of the T7 promoter to yield expression plasmid pVPET1. Similarly, DNA encoding PfF2 with a C-terminal six His fusion was amplified by PCR using primers 5'TCT AGT CCA TGG AAA AGC GTG AAC ATA TT3' and 5'ACG AGT GTC GAC TCA GTG ATG GTG ATG GTG ATG ATC GTC ATC ACG TTC TT3' and a plasmid containing the gene encoding *Plasmodium falciparum* EBA-175 as template. The PCR product was restricted with NcoI and SalI and cloned in the multiple cloning site of the vector pET28a+ downstream of the T7 promoter to yield plasmid pFPET1. *E. coli* BL21 (DE3) was transformed with plasmids pVPET1 and pFPET1 to yield the strains BL21 (DE3) pVPET1 and BL21(DE3) pFPET1, which were used for expression of recombinant PvRII and PfF2 respectively.

**Expression of PvRII and PfF2 in *E. coli*:** Luria broth (LB) medium containing kanamycin (50μg/ml) was inoculated with E. coli BL21 (DE3) pVPET1 or E. coli BL21 (DE3) pFPET1 and cultured overnight at 37°C. Fresh LB medium containing kanamycin (25μg/ml) was inoculated with the overnight culture at a dilution of 1:50 and cultured at 37°C to an $OD_{600nm}$ of 0.6-0.8. Expression of PvRII/PfF2 was induced by adding IPTG (iso propyl-1-thio-b-galactopyranoside) to the culture at a final concentration of 1mM and allowing the culture to grow for another 4 hours.

**Isolation of inclusion bodies:** Induced *E. coli* cultures were harvested by centrifugation and the cells were washed twice in chilled wash buffer A (10mM tris HCl pH 8.0,10mM EDTA, 100mM NaCl ). The washed cell pellet was resuspended in prechilled lysis buffer (10 mM Tris, pH 8.0, 5 mM Benzamidine-HCl, 2 mM PMSF, 10 mM EDTA, 100 mM NaCl, 200 µg/ml Lysozyme) and the cells were lysed by sonication. The lysed cells were centrifuged at 4°C, the supernatant was carefully decanted and the pellet containing inclusion bodies was retained. The inclusion bodies were washed twice with wash buffer B (10mM Tris-HCl, pH 7.2, 10mm EDTA, 100mM NaCl. 2-4M urea, 2% Triton X100) and once with 1 M NaCl.

**Purification of recombinant PvRII and PfF2 by metal affinity chromatography under denaturing conditions:** Purified inclusion bodies were dissolved in solublization buffer (10mM Tris HCl, pH 8.0, 100mM $NaH_2PO_4$, 100mM NaCl , 6M Guanidine–HCl, 10mM DTT) by stirring at 23°C overnight. Insoluble debris was removed by centrifugation and the supernatant was retained. Dithiothreitol was removed from the supernatant by filtration using a 10K cut off Amicon filter and Ni-NTA binding buffer (10mM Tris, pH 8.0, 100mM $NaH_2PO_4$, 6M Guanidine HCl). The solubilised inclusion bodies were loaded on a Ni-NTA column that had been equilibrated with equilibration buffer (10mM Tris pH 8.0, 100mM $NaH_2PO_4$, 6M guanidine-HCl). The column was washed with column wash buffer (10mM Tris pH 6.3, 100mM $NaH_2PO_4$, 6M guanidine-HCl) and the bound protein was eluted with elution buffer (10mM Tris pH 4.3, 100mM $NaH_2PO_4$, 6M guanidine-HCl). The final concentration of the purified protein was adjusted to around 4.5 mg/ml with elution buffer.

**Refolding PvRII and PfF2 by rapid dilution:** Ni-NTA purified PvRII and PfF2 were refolded by 100 fold rapid dilution in refolding buffer (50mM phosphate buffer - pH 7.2 for PvRII and pH 5.8 for PfF2, 1mM reduced glutathione (GSH), 0.1mM oxidised glutathione (GSSG), 1M urea, 0.5M arginine) to a final protein concentration of around 45 µg/ml. Refolding was allowed to proceed at 10°C for 36 hours with stirring. At the end of 36 hours, the refolding solution was dialyzed for

48 hours against dialysis buffer (50mM phosphate buffer - pH 6.5 for PvRII and pH 5.8 for PfF2, 1M urea) to remove arginine before proceeding with purification by ion-exchange chromatography.

The following ranges were tried:

Final protein concentration upon dilution: 10 μg/ml to 50 μg/ml

Arginine: 0.2M to 1M

Urea: 0.5M to 3M

Temp: 2-15°C

pH: 4.0 – 8.5

**Purification by ion-exchange chromatography and gel filtration chromatography:** Following removal of arginine by dialysis, the refolded protein was loaded on a SP-Sepharose column equilibrated with equilibration buffer (50mM phosphate buffer - pH 6.5 for PvRII and pH 5.8 for PfF2, 1M urea). The column was washed with equilibration buffer containing 100mM NaCl, 1 M urea. The bound protein is eluted with a linear gradient of equilibration buffer containing NaCl (100mM NaCl to 1.5 M NaCl). Fractions containing protein of interest were pooled and purified by gel filtration chromatography using a Superdex 75 column (Pharmacia, Sweden). During gel filtration chromatography 50 mM phosphate buffer (pH 7.2 for PvRII and pH 6.0 for PfF2) containing 200 mM NaCl were used.

**Analysis of refolded, purified PvRII and PfF2 by reverse phase chromatography:** Refolded PvRII and PfF2 were loaded on a reverse phase C8 column. The gradient used for elution was developed using Buffer A (0.05% TFA in water) and Buffer B (0.05% TFA in 90% acetonitrile, 10% water. The column was initially equilibrated with 90% Buffer A and 10% Buffer B and reached a composition of 10% Buffer A and 90% Buffer B in 40 minutes.

**Erythrocyte binding assay:** Refolded purified PvRII and PfF2 were added to normal or enzyme-treated RBCs and the reaction mix was incubated with rocking at room temperature for 1 hour. The reaction mixture was layered over oil and centrifuged to separate the RBCs with bound protein. Bound proteins were eluted from the RBCs with salt (300 mM NaCl) separated by SDS-PAGE and detected by

Western blot. Rabbit antisera raised against a peptide derived from PvRII and a mouse monoclonal antibody directed against the His tag were used to detect PvRII and PfF2 respectively.

**Rabbit antisera raised against refolded PvRII and PfF2 and assays for inhibition of RBC binding:** COS cells were transfected with a plasmid construct, pHVDR22, designed to express PvRII on the surface of mammalian cells as described earlier (5). Binding of RBCs to transfected COS cells expressing PvRII on the surface was tested using an erythrocyte-binding assay as described earlier (5). Erythrocyte binding assays were performed in the presence of different dilutions of rabbit antisera raised against refolded PvRII. The number of rosettes of COS cells covered with RBCs was scored in 50 fields at a magnification of 40.

**Mobility by gel electrophoresis and gel filtration chromatography:** Refolded PvRII and PfF2 had the expected mobility on SDS-PAGE gels (38 kD and 42 kD respectively) and were greater than 98% pure as determined by silver staining of SDS-PAGE gels (Figures 1, 2, 3). Recombinant PvRII was recognized in a Western blot by an antibody raised against a peptide derived from PvRII (Figure 1b). Recombinant PfF2 was recognized in a Western blot by an antibody directed against 5X-HIS fused to the C-terminal end of PfF2 (Figure 2b). The mobility of refolded PvRII and PfF2 was determined by SDS-PAGE before and after reduction with dithiothreitol (DTT) (Figure 4). Reduced PvRII and PfF2 had slower mobility on SDS-PAGE gels compared to non-reduced PvRII and PfF2 indicating the presence of disulfide linkages in the refolded proteins (Figure 4). The mobility of refolded PvRII and PfF2 was determined by gel filtration chromatography using a Superdex 75 (Pharmacia) column (Figure 5). Both PvRII and PfF2 had the expected mobility by gel filtration chromatography. No aggregates or oligomers were detected.

**Analysis of refolded PvRII and PfF2 by reverse phase chromatography:** Reverse phase chromatography can separate proteins based on differences in the hydrophobicity of exposed surfaces. Reverse phase chromatography (RP-HPLC) was used to determine if refolded PvRII and PfF2 contain heterogeneous populations with different conformers or a homogeneous population of native conformers. Both,

refolded PvRII andPfF2, elute as single, symmetric peaks on C-8 columns by reverse-phase chromatography indicating that they contain a single, homogeneous population of conformers (Figure 6).

**CD spectra:** The CD spectra of refolded and denatured PvRII were determined (Figure 7). As expected denatured PvRII did not show presence of any secondary structure. The negative peaks in the near UV range in the CD spectrum of refolded PvRII and PfF2 indicate presence of significant α helical structure. Refolded PvRII and PfF2 had similar CD spectra indicating that they have similar structures.

**Erythrocyte binding assay:** Refolded PvRII and PfF2 were tested for functional activity in erythrocyte binding assays (Figure 8). Refolded PvRII binds Duffy positive human RBCs but not Duffy negative human RBCs. Refolded PfF2 binds normal human and rhesus monkey RBCs but not neuraminidase-treated human or rhesus RBCs that lack sialic acid residues. Refolded PvRII and PfF2 thus bind RBCs with the correct specificity indicating that they are folded in their native conformations.

**Immunogenicity of refolded PvRII, ability to elicit blocking antibodies:** Refolded PvRII was used to immunize rabbits and determine if it is possible to elicit inhibitory antibodies. Sera from rabbits immunized with refolded PvRII and PfF2 were tested for reactivity with refolded PvRII and PfF2, respectively, using an ELISA (Figure 9). High titre rabbit antibodies that were directed against PvRII and PfF2 were detected by ELISA. The ability of these antisera to block the binding of PvRII to Duffy positive human erythrocytes was also tested. PvRII was expressed on the surface of mammalian COS cells as described earlier (Chitnis *et al*, 1994, J. Exp. Med **180**, p497-506) and tested for binding to human RBCs in the presence of different dilutions of rabbit antisera raised against refolded PvRII (Table 1). Rabbit antisera completely blocked binding of RBCs to PvRII up to a dilution of 1:2500. These data indicate that refolded PvRII is immunogenic and can elicit inhibitory antibodies capable of blocking the binding of PvRII to RBCs.

**Table II. Inhibition of RBC binding to PvRII expressed on COS cell surface with rabbit antibodies raised against refolded, recombinant PvRII.**

| Antisera | Dilution | No. of Rosettes in 50 Fields[a] |
|---|---|---|
| None | NA | 800 |
| Pre-immune | 1:10 | 850 |
| Adjuvant control | 1:10 | 600 |
| Anti-PvRII | 1:250 | 0 |
| Anti-PvRII | 1:500 | 0 |
| Anti-PvRII | 1:1,000 | 0 |
| Anti-PvRII | 1:2,500 | 0 |
| Anti-PvRII | 1:5,000 | 400 |
| Anti-PvRII | 1:7,500 | 450 |
| Anti-PvRII | 1:10,000 | 750 |

[a]Numbers of COS cells covered with rosettes of RBCs were scored in 50 fields at a magnification of 40. The average number of rosettes found in 3 wells is reported.

Pre-absorption of refolded PvRII with Duffy positive erythrocytes followed by detection of residual PvRII by immunoprecipitation and Western blotting was used to determine if some fraction of refolded PvRII is not functional. The sensitivity of detection of PvRII by immunoprecipitation followed by Western blotting was determined using known quantities of refolded PvRII. 10ng of refolded PvRII can be clearly detected by this method. Duffy positive and Duffy negative human erythrocytes were used to pre-absorb 8µg of refolded PvRII prior to immunoprecipitation. As expected, Duffy negative erythrocytes failed to absorb PvRII and residual PvRII was clearly detected. However, no residual PvRII was detected when Duffy positive erythrocytes were used for pre-absorption. Considering that the sensitivity of detection of PvRII was 100ng and 8µg of refolded PvRII was used, inability to detect residual protein after pre-absorption with Duffy positive erythrocytes indicates that at least 98.75% of refolded PvRII has been removed by pre-absorption and is functional (see Figure 10).

**Rabbit sera raised against refolded PfF2 and assays for inhibition of RBC binding**

Antibodies against PfF2 were raised in rabbits by immunization with refolded PfF2 formulated in Freund's adjuvant. Rabbit IgG antibodies were purified and tested for inhibition of erythrocyte invasion by *P. falciparum*. Late-stage schizonts of *P. falciparum* laboratory strain 3D7 and *P. falciparum* field isolate JDP8 were purified and incubated for 10 to 12 hours with human erythrocytes in the presence of different concentrations of purified rabbit antibodies raised against PfF2. Antibodies purified from rabbits immunized with adjuvant alone were used as control. Newly formed rings from fresh invasions were scored by Giemsa staining and efficiency of invasion was determined. Antibodies directed against PfF2 block erythrocyte invasion efficiently with around 95% inhibition at any antibody concentration of 1 mg/ml. Importantly, antibodies directed against PfF2 block invasion of both parasite strains tested. *P. falciparum* field isolate JDP8 invades human erythrocytes using multiple, sialic acid-independent pathways (Okoyeh J.N, Pillai C.R. and Chitnis C.E., 1999, *Infection and Immunity* **67**, p5784-5791). These data provide evidence that antibodies raised against PfP2 will effectively block erythrocyte invasion by diverse *P. falciparum* isolates providing support for vaccine development based on PfF2.

**Table III. Inhibition of RBC invasion with antibodies to PfF2**

| ***P. falciparum* strain** | **Antibody concentration (mg/ml)** | | | | |
|---|---|---|---|---|---|
| | 1 | 0.5 | 0.25 | 0.125 | 0.06 |
| 3D7 | 94.8% | 92.3% | 90.6% | 89.0% | 85.5% |
| JDP8 | 95.1% | 93.5% | 92.9% | 88.8% | 83.9% |

CLAIMS

1. A method of producing a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises:

(i) expressing the polypeptide in a bacterium, or as a non-secreted polypeptide in a yeast;

(ii) extracting the expressed polypeptide from the bacterium or yeast and denaturing the polypeptide;

(iii) refolding the extracted polypeptide in the presence of arginine and urea; and optionally

(iv) recovering the refolded polypeptide.

2. A method according to claim 1, wherein, in step (iii), multiple additions of the extracted polypeptide are made to the refolding solution.

3. A method according to claim 1, wherein the extraction is carried out under denaturing conditions.

4. A method according to claim 1 or 2, wherein in step (iii) the extracted protein is refolded by dilution with a solution containing the urea and arginine.

5. A method according to claim 3 wherein the dilution is from 50 to 100 fold, preferably 100 fold.

6. A method according to any one of the preceding claims wherein after the polypeptide has been refolded the recovery process includes removal of arginine and then subsequently removal of urea.

7. A method according to claim 6, wherein the arginine is removed by dialysis and/or the urea is removed by ion exchange chromatography or gel filtration chromatogrpahy.

8. A method according to anyone of the preceding claims wherein the ratio of arginine to urea in step (iii) is from 1:1 to 1:5.

9. A method according to claim 8 wherein the ratio is 1:2.

10. A method according to any one of claims 1 to 7, wherein in step (iii) the urea is present at a concentration of from 0.5 to 1.5 M and/or the arginine is present at a concentration of from 0.1 to 1 M.

11. A method according to claim 10, wherein the urea is present at a concentration of 1.0 M and the arginine is present at a concentration of 0.5 M.

12. A method according to any one of the preceding claims, wherein step (iii) is carried out at a temperature of from 5 to 15°C and/or for a period of from 12 to 48 hours.

13. A method according to claim 12, wherein step (iii) is carried out at a temperature of 10°C and/or for a period of 36 hours.

14. A method according to any one of the preceding claims, wherein the bacterium is *E. coli*.

15. A method according to anyone of the preceding claims, wherein the protein comprising a DBL domain is a *Plasmodium falciparum* or *Plasmodium vivax*, polypeptide.

16. A method according to claim 15, wherein the polypeptide is the *Plasmodium vivax* Duffy binding protein (PvDBP), the *Plasmodium falciparium* erythrocyte binding antigen (EBA-175), a PfEMP-1 family protein or a fragment variant or homologue of any of these comprising a DBL domain.

17. A method according to claim 16, wherein the polypeptide comprises region II (PvRII) of the PvDBP protein or region II (PfF2) of the EBA-175 protein or a fragment of either protein capable of specifically binding an erythrocyte.

18. A method according to claim 16 wherein the polypeptide has the amino acid sequence of SEQ ID NOs: 1 or 3 or a variant or fragment thereof capable of specifically binding the Duffy antigen or glycophorin.

19. A method according to anyone of the preceding claims wherein the polypeptide also comprises a member of a specific binding pair to allow its recovery.

20. A method according to claim 19, wherein the member of a specific binding pair is a His tag.

21. A method according to any one of the preceding claims, wherein the extracted polypeptide is recovered by extracting inclusion bodies from the bacterium or yeast and recovering the polypeptide from said inclusion bodies.

22. A method according to any one of the preceding claims further comprising formulating the refolded polypeptide into a pharmaceutical composition.

23. A method according to claim 22, wherein the pharmaceutical composition is a vaccine composition.

24. A pharmaceutical composition obtainable or obtained by the method of claim 22.

25. A vaccine composition obtainable or obtained by the method of claim 23.

26. A method for the identification of a substance that modulates the interaction between a polypeptide produced by a method as defined in any one of claims 1 to 21 and a host cell receptor involved in the entry of a parasite into a host cell, wherein the method comprises:

(i) contacting the receptor with the polypeptide in the presence of a test substance;

(ii) determining the effect of the test substance on the interaction between the receptor and polypeptide thereby to determine whether the test substance is capable of modulating the interaction between the receptor and polypeptide.

27. A method according to claim 26, wherein the receptor is present on the surface of a cell.

28. A method of treating or preventing malaria in an individual comprising immunising the individual with a pharmaceutical composition according to claim 22 or a vaccine composition according to claim 23.

29. Use of a substance identified by a method according to claim 26 or 27 in the manufacture of a medicament for treating or preventing malaria.

Fig.1A.

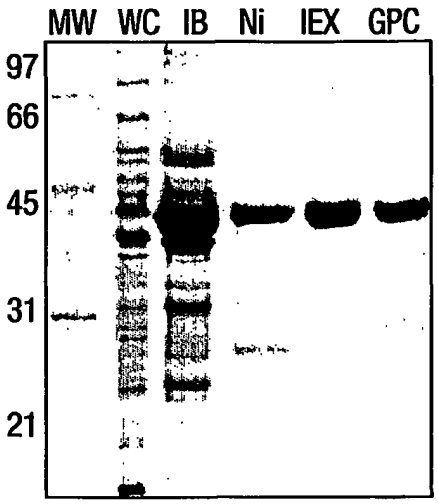



Fig.1B.

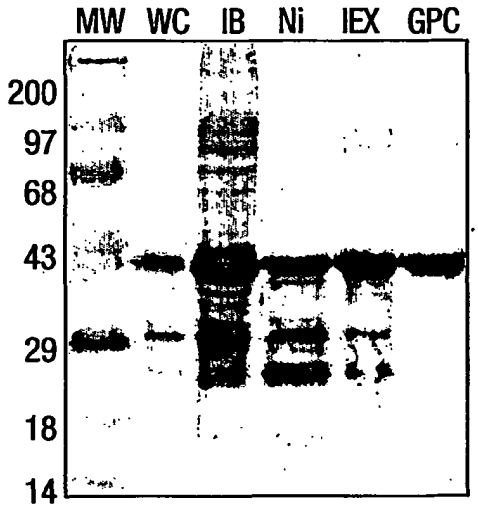



Fig.2A.

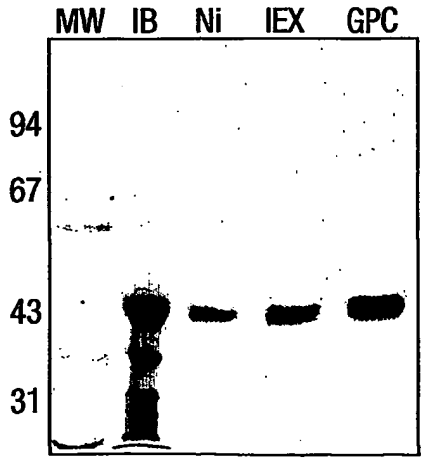



Fig.2B.

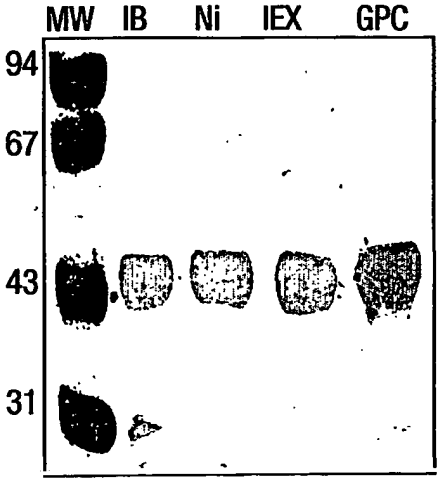


Fig.3A.

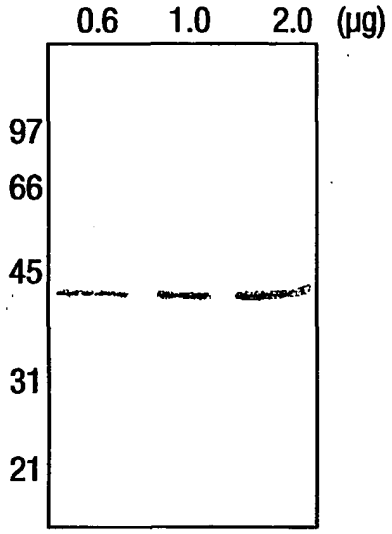



Fig.3B.

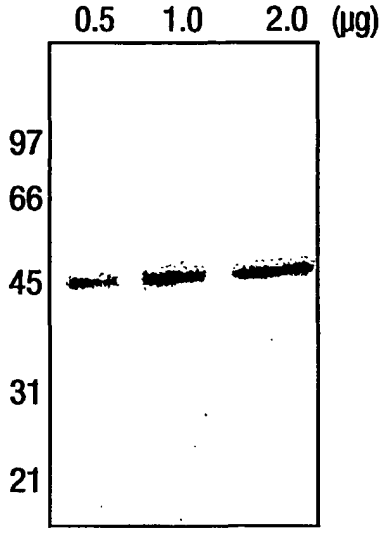



Fig.4A.

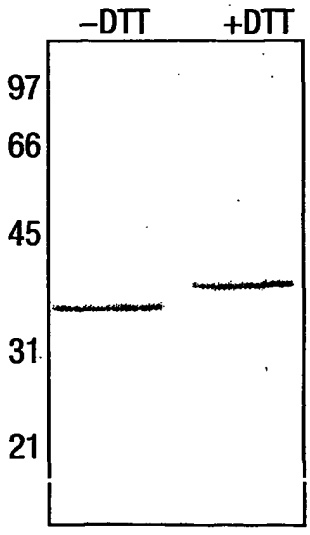
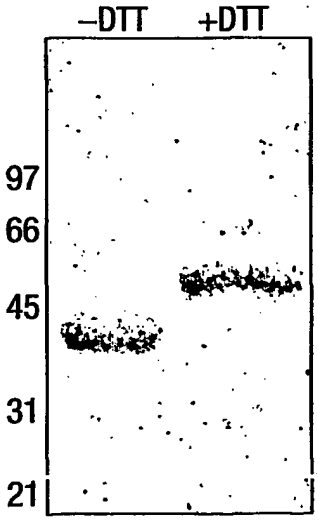
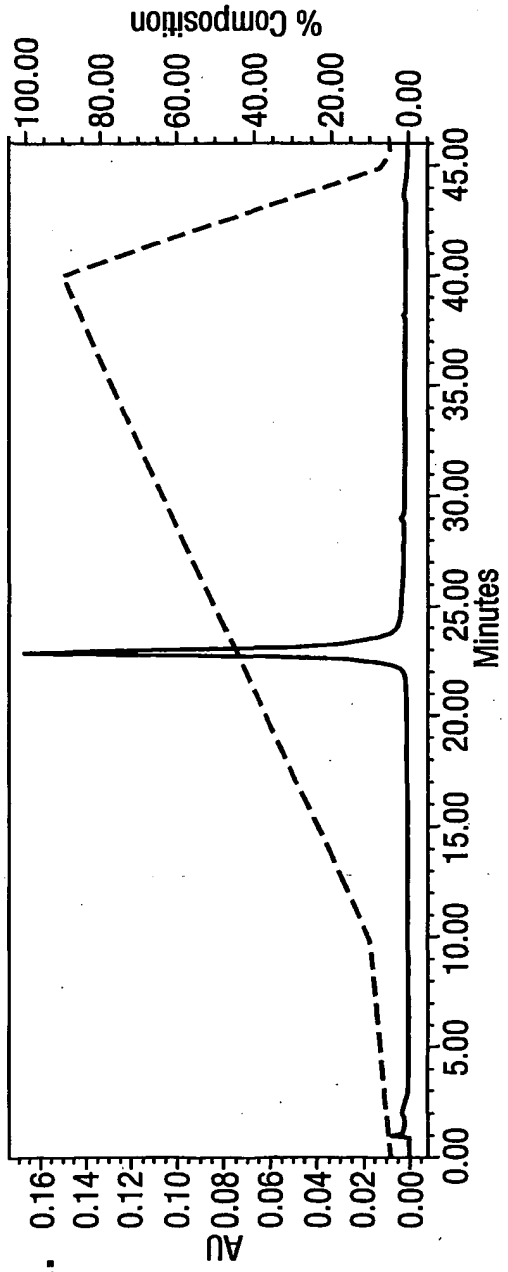



Fig.4B.

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0.02
0.00
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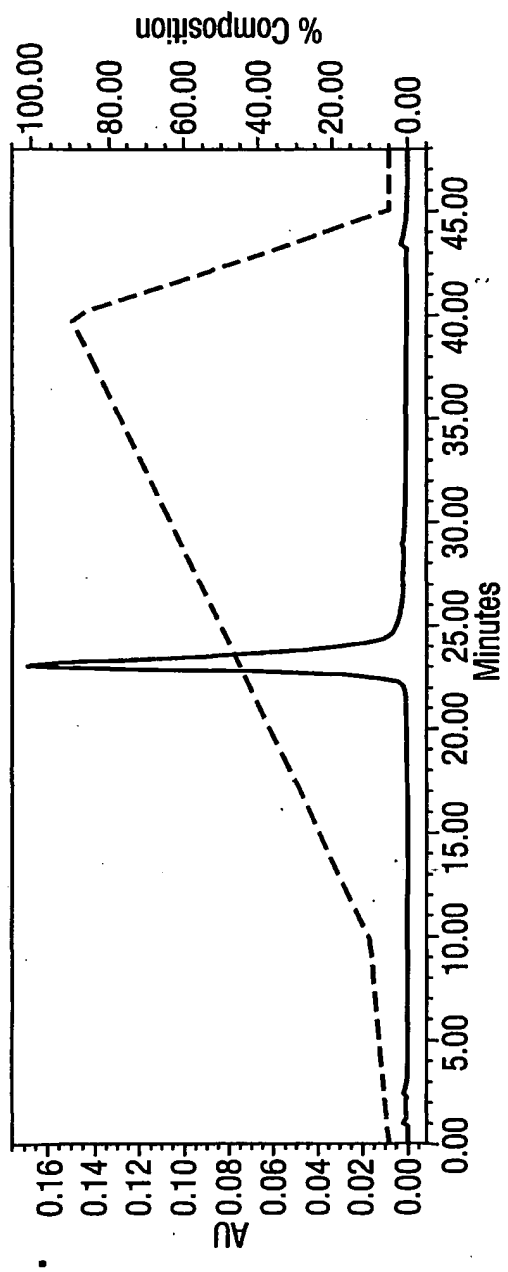
45.00
Minutes

Fig.5A.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Minutes

Fig.5B.

Fig.6A.

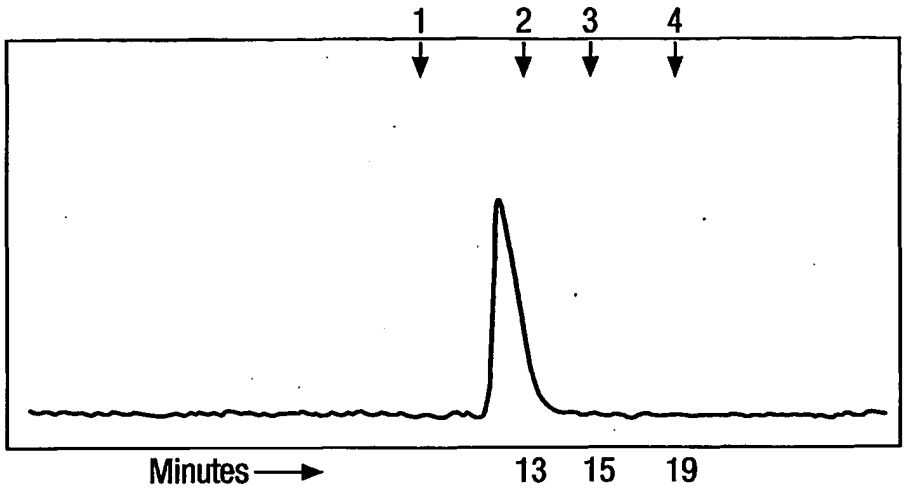



Fig.6B

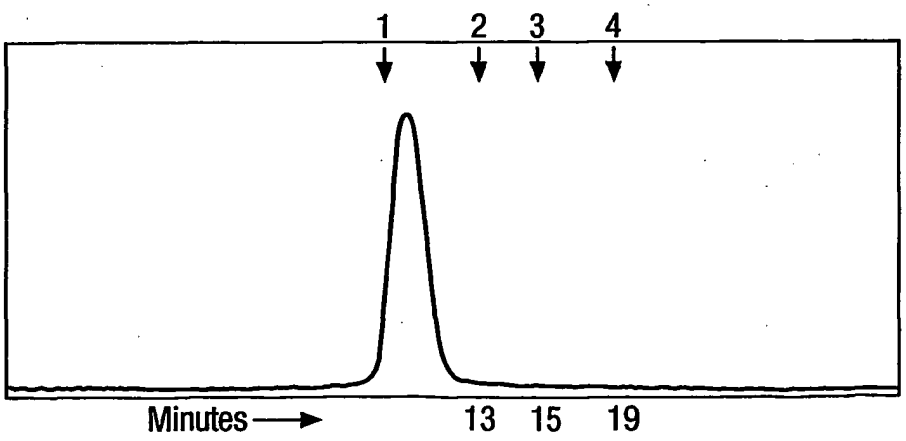


Fig.7B.

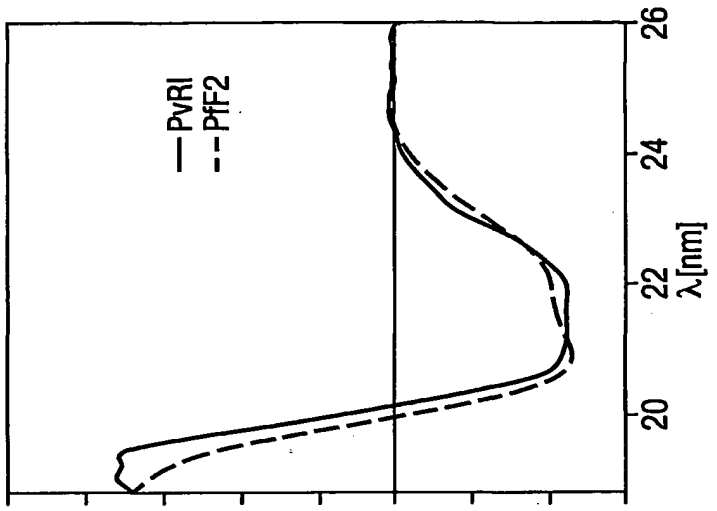
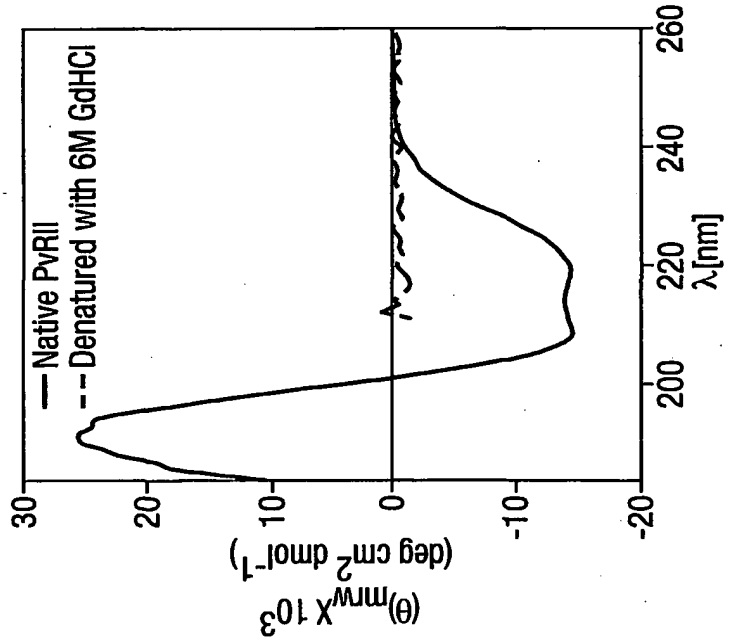

PvRI
PfF2
20
22
24
26
λ[nm]

Fig.7A.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Denatured with 6M GdHCl
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20
10
0
-10
-20
200
220
240
260
λ[nm]
$(\theta)_{mrw} \times 10^3$
(deg cm$^2$ dmol$^{-1}$)

Fig.8A.

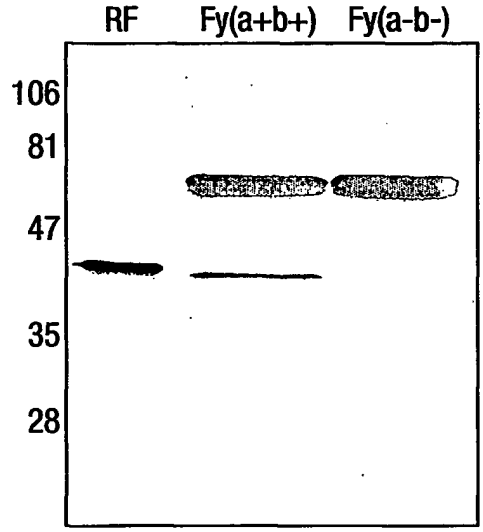



Fig.8B.

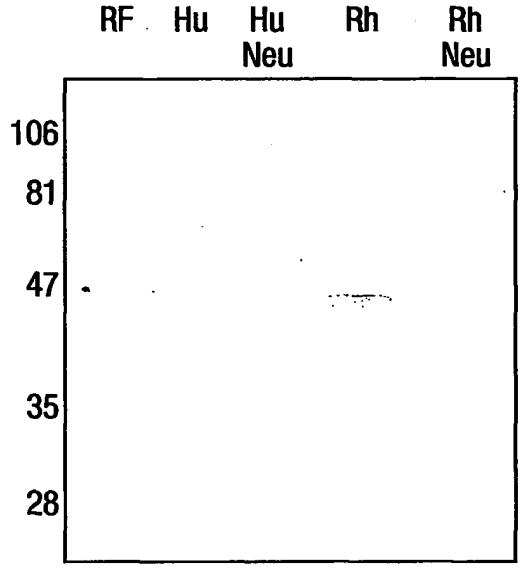
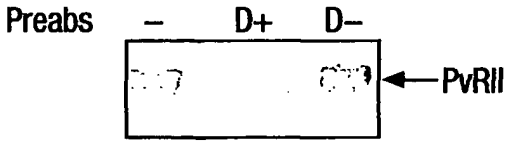



Fig.10.



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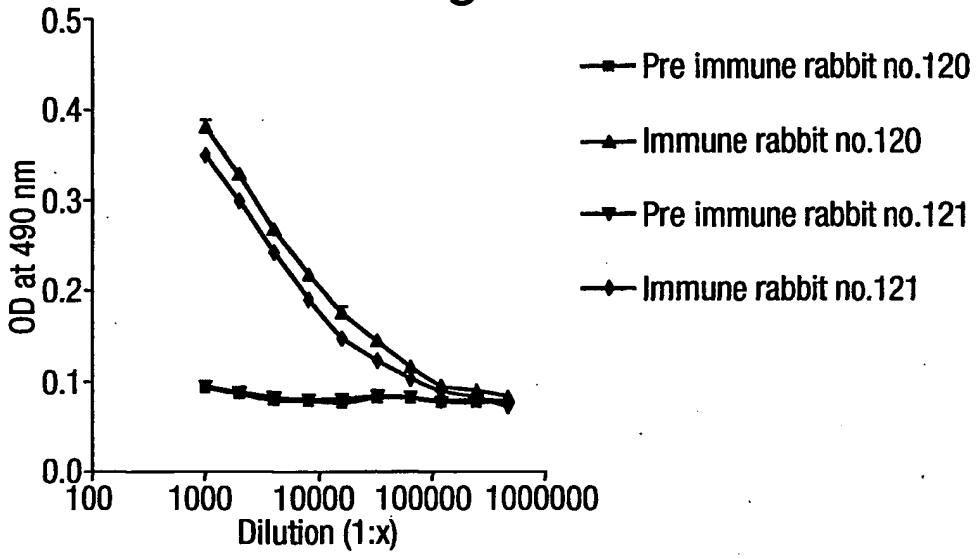
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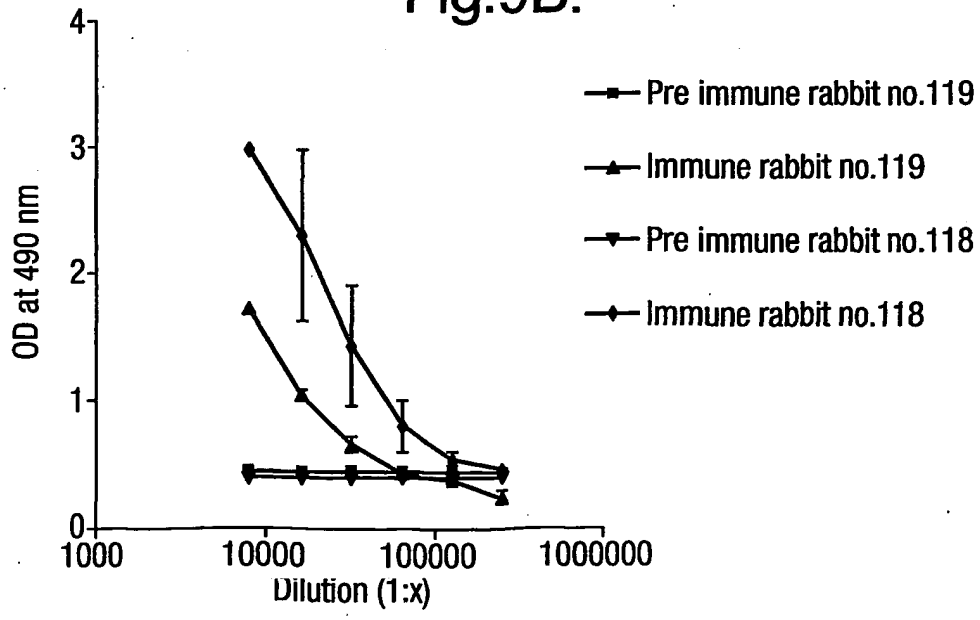
Immune rabbit no.120
Pre immune rabbit no.121
Immune rabbit no.121

Fig.9B.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# SEQUENCE LISTING

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| Leu | Gln | Asn | Thr | Val | Met | Lys | Asn | Cys | Asn | Tyr | Lys | Arg | Lys | Arg | Arg |
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165 170 175
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Glu Thr Gln Lys Val Pro Lys Glu Asn Ala Glu Asn Tyr Leu Ile Lys
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50 55 60

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65 70 75 80

Ile Leu Lys Arg Lys Tyr Lys Asn Lys Asp Asp Lys Glu Val Cys Lys

85 90 95

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100 105 110

Asp Tyr Trp Asn Asp Leu Ser Asn Arg Lys Leu Val Gly Lys Ile Asn

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